University of Groningen

Metabolic Energy Generation In Hydrogenosomes Of The Anaerobic Fungus Neocallimastix - Evidence For A Functional-relationship With Mitochondria

Marvin-Sikkema, F. D.; Driessen, A. J. M.; Gottschal, J. C.; Prins, R. A.

Published in:
Mycological Research

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1994

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Marvin-Sikkema, F. D., Driessen, A. J. M., Gottschal, J. C., & Prins, R. A. (1994). Metabolic Energy Generation In Hydrogenosomes Of The Anaerobic Fungus Neocallimastix - Evidence For A Functional-relationship With Mitochondria. *Mycological Research*, *98*, 205-212.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Metabolic energy generation in hydrogenosomes of the anaerobic fungus *Neocallimastix*: evidence for a functional relationship with mitochondria

FEMKE D. MARVIN-SIKKEMA*, ARNOLD J. M. DRIESSEN, JAN C. GOTTSCHAL AND RUDOLF A. PRINS

Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Anaerobic eukaryotes are often devoid of mitochondria but contain special organelles separated from the cytosol by a single (in fungi) or a double (in protozoa) membrane. Hydrogenosomes from the anaerobic fungus *Neocallimastix* sp. L2 are thought to catalyse the enzymic steps in the ATP-yielding metabolism of malate into acetate, H_2 and CO_2. Isolated hydrogenosomes contain a Mg^{2+}- or Mn^{2+}-dependent ATPase activity. This activity is involved in the maintenance of a pH gradient across the hydrogenosomal membrane, which renders these organelles alkaline inside. ATPase activity and ΔpH generation is sensitive to diethylstilboestrol but not to other known ATPase inhibitors. Typical inhibitors of the mitochondrial ADP/ATP translocase, bongkrekic acid and carboxyatractylate reduced the ATPase activity, suggesting the presence of a nucleotide transporter. Under anaerobic conditions hydrogenosomes produced H_2 and acetate from malate. This process was found to be dependent on the external supply of ATP or ADP and succinate, and was blocked by protonophores, diethylstilboestrol, and the inhibitors bongkrekic acid and carboxyatractylate. These results demonstrate that hydrogenosomes of *Neocallimastix* sp. L2 perform the essential functions required for the generation of metabolic energy from malate. It is suggested that hydrogenosomes are functionally related to mitochondria but lack an outer membrane.

Anaerobic fungi are important inhabitants of the digestive tract of many herbivorous mammals (Orpin & Joblin, 1988; Milne *et al.*, 1989). They play a role in the degradation of plant material by the production of cell wall-degrading enzymes (Williams & Orpin, 1987) and by physically weakening the cell wall structure with their rhizoidal system (Akin *et al.*, 1989). Anaerobic fungi and many other anaerobic eukaryotes, such as trichomonad protozoa and (rumen) ciliates, contain special organelles, termed 'hydrogenosomes'. A major share of the metabolism leading to the production of H_2, CO_2 and acetate is thought to be localized in these organelles (Marvin-Sikkema *et al.*, 1993*b*; Müller, 1988; Yarlett *et al*, 1986). Characterization of the metabolic functions of these organelles is impaired by the liability to molecular oxygen and the difficulty of obtaining stable and intact hydrogenosomes. The recent development of a rapid procedure for the isolation of intact hydrogenosomes from the anaerobic fungus *Neocallimastix* sp. L2 (Marvin-Sikkema *et al.*, 1993*b*) enables us for the first time to study the function of these organelles in detail. When grown on glucose as sole energy source, *Neocallimastix* sp. L2 produces H_2 and CO_2. These products are presumed to be formed from malate, which is thought to be a substrate for hydrogenosomes (Marvin-Sikkema *et al.*, 1993*b*). Hydrogenosomal metabolism also leads to the production of ATP at substrate level. The energy-generating function of the hydrogenosomes is further emphasized by their localization in the vicinity of the flagellar pole in the zoospores of *Neocallimastix* sp. L2 (Marvin-Sikkema *et al.*, 1992, 1993*b*). Hydrogenosomes may present the energy to the flagellar motors required for locomotion. Mitochondria are absent in this organism (Marvin-Sikkema *et al.*, 1992), and are possibly replaced by the hydrogenosomes, suggesting a functional relationship between these organelles. On the other hand, a structural and functional relationship with microbodies from aerobic eukaryotes (Marvin-Sikkema *et al.*, 1993*a*, *b*) has also been suggested as the hydrogenosomes bear only a single membrane (Marvin-Sikkema *et al.*, 1992), while immunological studies suggest that some hydrogenosomal proteins harbour the universal microbody protein targeting signal SKL (Marvin-Sikkema *et al.*, 1993*a*).

As hydrogenosomes produce ATP, the question is raised whether these organelles maintain a protonmotive-force (Δp) across the membrane at the expense of ATP. The Δp may then be utilized for protein import and transport of substrates and/or products into and out of the hydrogenosome. The presence of transmembrane pH gradient (ΔpH) may create an optimal environment for the functioning of hydrogenosomal enzymes. Synthesis of ATP inside hydrogenosomes furthermore requires a transport mechanism which allows entry and exit of nucleotides into and out of the hydrogenosomal lumen.

In this paper we show the presence of a hydrogenosomal

* Corresponding author. Present address: Division of Industrial Microbiology, Department of Food Science, Wageningen Agricultural University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands.

ATPase which is responsible for the generation of a ΔpH across the hydrogenosomal membrane of *Neocallimastix* sp. L2, rendering the lumen of these organelles alkaline *v.* the cytosol. Indirect evidence provided by the effect of inhibitors and ion- and protonophores on H_2-production by intact hydrogenosomes under anaerobic conditions suggests the presence of an ADP/ATP translocase. It is concluded that hydrogenosomes of *Neocallimastix* sp. L2 represent novel organelles sharing structural and functional properties with mitochondria and microbodies.

MATERIALS AND METHODS

Chemicals

Bis-(3-propyl-5-oxoisoxazol-4-yl) pentamethine (oxonol VI), 2′,7′-bis-(2-carboxyethyl)-5 (and 6)-carboxyfluorescein acetoxymethylester (BCECF), and 9-amino-6-chloro-2-methoxyacridine (ACMA) were obtained from Molecular Probes Inc., Eugene, OR, U.S.A. Carboxy-[^{14}C]-benzoic acid (50 mCi mmol^{-1}) was obtained from Amersham, Buckinghamshire, United Kingdom. Bongkrekic acid was a generous gift from Dr Reinard Krämer (Institut für Biotechnologie, Jülich, Germany). Bafilomycin A_1 was a gift from Dr Karl-Heinz Altendorf (University of Osnabrück, Osnabrück, Germany). All other chemicals were reagent grade and obtained from commercial sources.

Organism and cultivation

Neocallimastix sp. L2 (Marvin-Sikkema *et al.*, 1990, 1992) was cultured at 39 °C in 16 ml Hungate tubes fitted with screw caps and butyl rubber septa (Bellco Glass Inc., Vineland, NJ, U.S.A.), containing 10 ml aliquots of medium, or in 1 l serum bottles with butyl rubber stoppers in a defined medium supplemented with 20 mM glucose (Marvin-Sikkema *et al.*, 1990). Tubes and bottles were inoculated with 0·5–2·0 ml of culture pre-grown for 48 h in the same medium. Cultures were routinely transferred every 2–3 d.

Preparation of cell-free extract

Cell-free extract was prepared in 20 mM-K-HEPES, pH 7·4, 2 mM-DTT as described before (Marvin-Sikkema *et al.*, 1993*b*).

Cell fractionation

Hydrogenosomes of *Neocallimastix* sp. L2 were isolated as described elsewhere (Marvin-Sikkema *et al.*, 1993*b*), but the potassium phosphate (KP_i) in the buffers was replaced by 20 mM-K-HEPES, pH 7·4.

Anaerobic incubation of hydrogenosomes with malate or pyruvate

A hydrogenosome-enriched pellet was obtained as described before (Marvin-Sikkema *et al.*, 1993*b*), using K-HEPES instead of KP_i buffer. The pellet was suspended in 3ml K-HEPES, pH 7·4, 250 mM sucrose, 2 mM DTT. The hydrogenosome suspension (100 µl containing approximately 50 µg of protein) was incubated at 39° in 7 ml crimp-seal bottles with butyl rubber septa with the following solutions (final volume 2 ml): 20 mM-K-HEPES, pH 7·4, 250 mM sucrose, 5 mM L-malate or pyruvate, 2 mM-KP_i, pH 7·4, 100 µM succinate, 5 mM-MgATP or MgADP. The mixture was flushed with N_2 before the addition of the hydrogenosome suspension. At regular intervals samples were withdrawn for analysis of malate, pyruvate and acetate, and the gas phase was checked for H_2 production. The influence of CO_2 on H_2 production was examined in the same mixture with an additional 20 mM-$NaHCO_3$. The mixture was flushed with 20% CO_2/80% N_2 instead of 100% N_2.

Enzyme assays

All enzyme assays were carried out anaerobically at 39°. The activity of hydrogenase (EC 1.18.3.1) was assayed as described by Marvin-Sikkema *et al.* (1993*b*), adenylate kinase (EC 2.7.4.3) as described by Declerck & Müller (1986), and 5′-nucleotidase with a colorimetric method as described by Widnell (1974). ATPase activity was determined by both continuous and discontinuous assays, as described by Douma *et al.* (1987). Alkaline phosphatase activity was determined as described by Yarlett *et al.* (1986). Protein was determined with the Bio-Rad assay system (Bio-Rad, Watford, U.K.) with bovine serum albumin as a standard. Sucrose densities were estimated by measurements of the refractory index.

Determination of the pH gradient

The presence of a pH gradient across the hydrogenosomal membrane was examined by fluorescent methods using ACMA and BCECF. Fluorescence was measured in a Perkin Elmer LS50 spectrofluorimeter with a temperature-controlled cuvette at 39° and computer-controlled data acquisition.

ACMA fluorescence quenching was recorded at 474 nm using an excitation wavelength of 409 nm. The reaction mixture contained 20 mM-K-HEPES, pH 7·4, 250 mM sucrose, 10 mM creatine phosphate, 10 µg ml^{-1} creatine kinase, 15 mM-ACMA, and hydrogenosomes (approximately 250 µg protein ml^{-1}).

For BCECF fluorescence, hydrogenosomes (500 µg protein) were incubated for 30 min at room temperature with the acetoxymethyl ester of BCECF (50 µM) and washed twice with 20 mM-K-HEPES, pH 7·4 and 250 mM sucrose to remove non-entrapped BCECF. The presence of BCECF in hydrogenosomes was checked by fluorescence microscopy. Fluorescence was recorded at 525 nm after excitation at 500 nm in the reaction mixture as described above with BCECF-loaded hydrogenosomes (approximately 250 µg protein ml^{-1}). Hydrogenosomes were stored on ice until use, and reactions were started by dilution of the hydrogenosomal suspension into buffer. Carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) (10 µM) was used for the dissipation of the ΔpH. Calibration of BCECF fluorescence was performed by measuring the fluorescence at different pH values in non-energized hydrogenosomes after dissipation of the pH gradient by CCCP, i.e. conditions of equal external and internal pH.

Determination of the $\Delta\psi$

The transmembrane electric potential ($\Delta\psi$, inside negative) was measured by the distribution of tetraphenylphosphonium ion (TPP^+) using an ion-selective electrode (de Vrij *et al.*, 1986) at 39°. The mixture contained 20 mM-K-HEPES, pH 7·4, 250 mM sucrose, 25 mM-K_2SO_4, 2 mM-MgATP, 10 mM creatine phosphate, 10 μg/ml creatine kinase, 3·3 mM-TPP^+ and hydrogenosomes (approx. 250 μg protein). Valinomycin (5 μM) was used for the dissipation of the $\Delta\psi$. $\Delta\psi$ (inside positive) was measured by oxonol VI fluorescence quenching. The reaction mixture was the same as described above, but TPP^+ was omitted and 2 mM oxonol VI was added. Oxonol VI fluorescence quenching was measured at 634 nm after excitation at 599 nm, at 39°.

Analytical methods

Acetate was analysed by gas chromatography (Nanninga, Drent & Gottschal, 1986), H_2 and CO_2 were determined as described by Gerritse, Schut & Gottschal (1990). Pyruvate was determined in an assay mixture, containing 100 mM-KP_i, pH 6·2, 0–0·1 mM pyruvate, 0·2 mM-NADH, and 10 U ml^{-1} L-lactate dehydrogenase. Samples were incubated at 30° and the decrease in absorbance at 340 nm was taken as a measure for the pyruvate concentration. Malate was assayed after Gutman & Wahlefeld (1974).

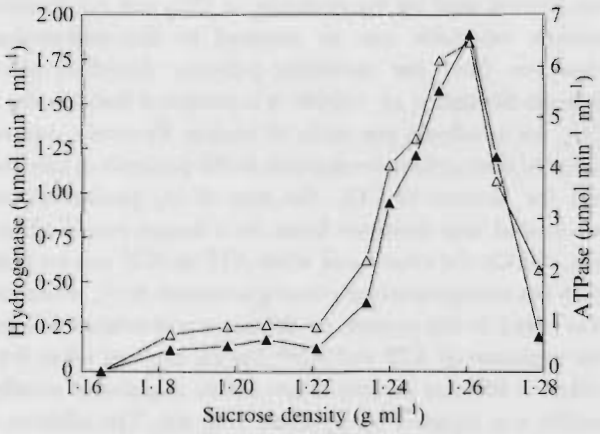

Fig. 1. Hydrogenase- and ATPase-activity patterns obtained after sucrose density centrifugation of a hydrogenosome-enriched fraction of *Neocallimastix* sp. L2. ▲, Hydrogenase activity; △, ATPase activity.

Table 1. Properties of the hydrogenosomal ATPase of *Neocallimastix* sp. L2

pH optimum	8·5
K_m for ATP	1·2 mM
Cation dependency	Mn^{2+} or Mg^{2+} No Ca^{2+} or Co^{2+}
Inhibited by	Diethylstilboestrol (2 μM)
Not inhibited by	Dicyclohexylcarbodiimide (10 μM), oligomycin (10 μg ml^{-1}), azide (3 mM), vanadate (1·5 mM), bafilomycin A_1 (100 μg ml^{-1}), nitrate (5 mM)

RESULTS

Hydrogenosomes contain a novel ATPase

A fraction enriched in hydrogenosomes is conveniently obtained after differential centrifugation of a cell homogenate of *Neocallimastix* sp. L2 (Marvin-Sikkema *et al.*, 1993*b*). The 30000 ***g*** pellet contained substantial ATPase activity, i.e. 5·4 μmol min^{-1} mg $protein^{-1}$ when measured in the presence of an ATP-generating system (see below). After sucrose density centrifugation of this fraction, activity peaks of the ATPase and hydrogenase, a marker enzyme of the hydrogenosomes (Marvin-Sikkema *et al.*, 1993*b*) coincided at a sucrose density of 1·25–1·26 g ml^{-1} (Fig. 1).

Electron microscopical observation demonstrates that at this density, highly purified hydrogenosomes are present (Marvin-Sikkema *et al.*, 1993*b*). The ATPase activity was not due to alkaline phosphatase or 5′-nucleotidase activities, as these were absent in the hydrogenosomal fractions. The absence of 5′-nucleotidase activity indicates that the observed activity is not caused by contamination with plasma membranes (Widnell, 1974). Adenylate kinase activity is also not responsible for the observed ATPase activity. P^1P^5-di(adenosine 5′-) pentaphosphate, a specific inhibitor of adenylate kinase, did not influence the ATPase activity. This inhibitor completely blocked the adenylate kinase activity of the hydrogenosomes at a concentration of 100 μM (not shown).

Fractions from the sucrose gradient with sucrose densities of 1·25–1·26 g ml^{-1} were pooled and used to study the properties of the hydrogenosomal ATPase (Table 1). Of the known specific ATPase inhibitors, only diethylstilboestrol (DES) was found to block the ATPase activity (Table 1). Typical mitochondrial ATPase inhibitors such as N,N′-dicyclohexylcarbodiimide (DCCD) and oligomycin were without effect. When ADP was present in the assay mixture, the ATPase activity was lowered. This is also evident from the observations that the use of an ATP-regenerating system (PEP + pyruvate kinase) significantly elevated the 'apparent' ATPase, i.e. 4·8 instead of 0·8 μmol min^{-1} $protein^{-1}$. When the ATPase assay was carried out with intact hydrogenosomes osmotically stabilized in 250 mM sucrose, the activity was reduced by the ADP/ATP translocase inhibitors bongkrekic acid (1 or 5 μM) or carboxyatractylate (10 μM) to about one-third of the activity measured in the absence of these compounds, i.e. 1·3 and 4·8 μmol min^{-1} mg $protein^{-1}$, respectively. These inhibitors had no effect on the ATPase activity when assayed in the presence of 0·1% (v/v) Triton X-100 (not shown). These data suggest that hydrogenosomes contain an ATPase activity localized in the organelle.

Hydrogenosomal lumen is alkaline v. outside

Fluorescence microscopy indicated that hydrogenosomes of *Neocallimastix* sp. L2 can be efficiently loaded with the water-soluble fluorescence pH indicator BCECF when incubated with the membrane-permeable acetoxymethyl ester. This implies that the hydrogenosomes contain an esterase which is involved in the hydrolysis of the BCECF–ester bond. Hydrogenosomes properly retained the entrapped BCECF when stored on ice (not shown). Fluorescence measurements

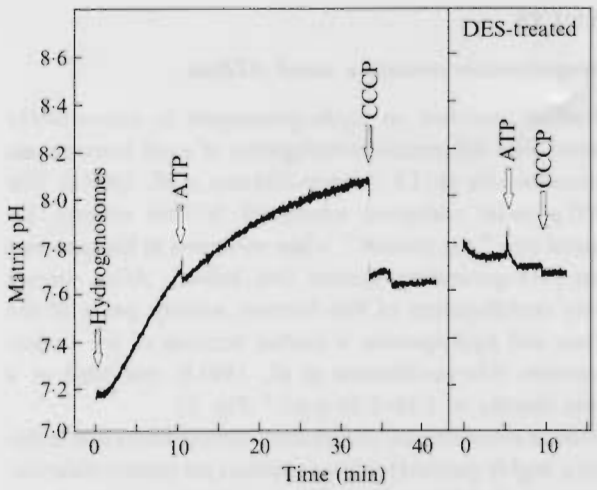

Fig. 2. Matrix pH traces of BCECF-loaded hydrogenosomes from *Neocallimastix* sp. L2. The hydrogenosomes were incubated for 10 min with 2 μM diethylstilboestrol prior to the experiment. ATP (2 mM) and CCCP (10 μM) were added at the times indicated. Loading of the hydrogenosomes with the acetoxymethyl ester of BCECF and the calibration of BCECF fluorescence quenching were performed as described in Materials and Methods.

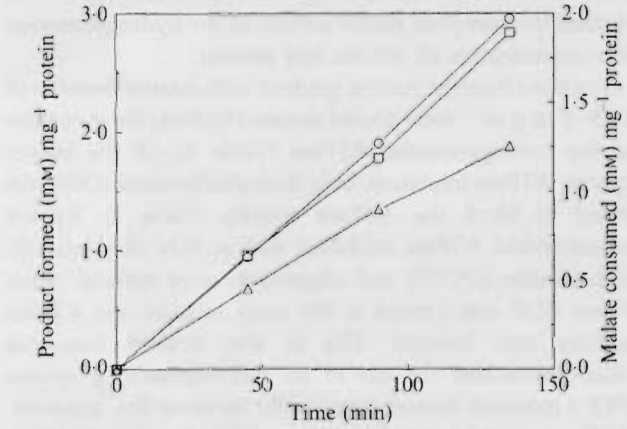

Fig. 3. H_2 and acetate production and malate consumption by isolated hydrogenosomes from *Neocallimastix* sp. L2. Incubations were carried out in the presence of 5 mM-MgATP as described in Materials and Methods. □, H_2; △, acetate; ○, malate.

with BCECF-loaded hydrogenosomes, diluted in 20 mM-K-HEPES, pH 7·4, 250 mM sucrose, indicated that hydrogenosomes were internally alkaline (Fig. 2). Addition of ATP had no effect on the development of the BCECF fluorescence, while a rapid decrease of the fluorescence level was observed upon the addition of the protonophore CCCP (10 μM) (Fig. 2). The combination of valinomycin (5 μM) and nigericin (100 nm) in the presence of 25 mM K_2SO_4 had no effect on the ΔpH (not shown). The ΔpH was completely absent in hydrogenosomes pre-incubated with 2 μM-DES (Fig. 2), while other ATPase inhibitors had no effect (not shown). These results suggest that the isolated hydrogenosomes maintain a pre-existing pH gradient which is presumably formed at the expense of residual ATP present in the intact hydrogenosomes. The inhibition by DES suggests that the hydrogenosomal ATPase is involved in the generation of ΔpH. When the ΔpH was measured with alternative methods, i.e. flow dialysis measuring the distribution of benzoic acid (Hellingwerf & Konings, 1980) and fluorescence quenching measurements with the fluorescent amine ACMA, essentially similar results were obtained (not shown). In this respect, an increase in ACMA fluorescence was found which was reversed by CCCP, lending further support to the conclusion that the hydrogenosomes maintain a pH gradient across the membrane, alkaline inside versus outside.

The presence of a Δψ across the hydrogenosomal membrane was investigated with the fluorescence dye oxonol VI (Δψ, inside positive) and the lipophilic cation TPP^+ (Δψ, inside negative). No evidence was obtained that the isolated, resting, organelles maintained a Δψ which is above the detection limit of these methods of about −30 mV.

Hydrogen production by hydrogenosomes requires an external supply of nucleotides and is blocked by protonophores

Under anaerobic conditions, isolated hydrogenosomes produce H_2 and acetate when incubated with malate (Fig. 3), while the introduction of molecular oxygen immediately blocked metabolism (data not shown). Malate is stoichiometrically converted into acetate with the simultaneous production of 1·5 mole of H_2 per mole of malate. These values are in agreement with our suggestions for the *in vivo* situation in *Neocallimastix* sp. L2 (Marvin-Sikkema *et al.*, 1993*b*). Since the method used for the detection of CO_2 was not sensitive enough, no value van be assigned to this end-product. However, from the metabolic pathway described before (Marvin-Sikkema *et al.*, 1993*b*), it is predicted that 2 moles of CO_2 are produced per mole of malate. Pyruvate was not detected during these incubations. In the presence of succinate and the absence of CO_2, the rate of H_2 production was accelerated, and remained linear for a longer period of time (Fig. 4*a*). On the other hand, when ATP or ADP was excluded from the reaction mixture a strong reduction in H_2 production was noted. In this respect, no difference was evident between the exclusion of ATP and ADP. No H_2 evolved when 0·1% Triton X-100 was present, when malate was absent or when malate was replaced by pyruvate (Fig. 4*a*). The addition of external ferredoxin did not result in a restoration of H_2 production by lysed hydrogenosomes (not shown). These data show that intact hydrogenosomes catalyse the imperative enzymic steps required for the conversion of malate into acetate. Next, we determined the effect of metabolic inhibitors that interfere with putative membrane functions. The ionophores valinomycin and/or nigericin, and the protonophore CCCP strongly interfered with H_2 production (Fig. 4*b*). The ATPase inhibitor diethylstilboestrol also lowered H_2 production (Fig. 4*c*), while vanadate and DCCD, which had no effect on the hydrogenosome-associated ATPase activity, were without effect. A reduction in the rate of H_2 production was also evident with the adenylate kinase inhibitor P^1P^5-di-(adenosine 5′-) pentaphosphate, although the effect was not as pronounced as found for the ionophores and the ATPase inhibitor. At low concentrations, bongkrekic acid and carboxyatractylate, both specific inhibitors of the mito-

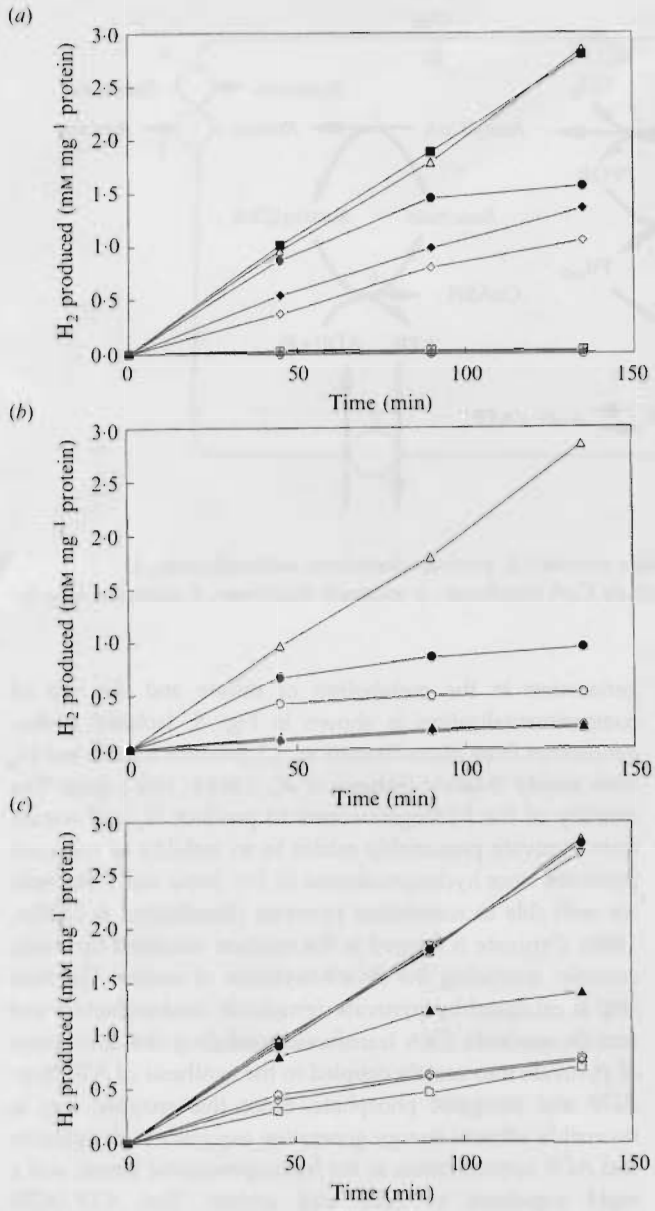

Fig. 4. Influence of several compounds on H_2 production from malate by isolated hydrogenosomes from *Neocallimastix* sp. L2. (*a*) Incubation carried out under standard conditions (△), with 5 mM-MgADP instead of MgATP (■), in the absence of succinate (●), in the presence of CO_2 (◆), in the absence of MgATP (◇), in the absence of malate (○), with pyruvate instead of malate (□), and in the presence of 0·1% Triton X-100 (+). (*b*). Incubation carried out under standard conditions (△), in the presence of 5 μM valinomycin (●), 100 nM nigericin (○), 5 μM valinomycin and 100 nM nigericin (▲), or 10 μM-CCCP (◇). (*c*) Incubation carried out under standard conditions (△), in the presence of 10 μM-DCCD (▽), 1·5 mM vanadate (●), 100 μM P^1P^5-di(adenosine-5′) pentaphosphate (▲), 2 μM diethylstilboestrol (□), 1 μM bongkrekic acid (○) or 10 μM carboxyatractylate (◇). Incubations and analyses were performed as described in Materials and Methods, and reactions contained 5 mM-MgATP unless stated otherwise.

chondrial ATP/ADP translocase, strongly inhibit H_2 production to about the level in the absence of externally added nucleotides. These data suggest that H_2 production from malate requires the presence of nucleotides in the lumen of the hydrogenosomes, and that these nucleotides are transported into and out of the hydrogenosomes by a transport protein that, as far as its function concerns, resembles the mitochondrial ATP/ADP translocase.

DISCUSSION

In this paper we have demonstrated that hydrogenosomes from the anaerobic fungus *Neocallimastix* sp. L2 fulfil an energy-generating function in the anaerobic metabolism of glucose. These organelles metabolize malate into acetate, H_2 and CO_2, a reaction which is coupled to the production of ATP from ADP and inorganic phosphate at substrate level. Our data suggest that cycling of the nucleotides into and out of the hydrogenosomes is important for malate metabolism, possibly mediated by an ATP/ADP exchange transport protein. Hydrogenosomes possess an ATPase which may be involved in the maintenance of a protonmotive force across the membrane. Δp may function in the transport of metabolites into and out of the hydrogenosome and provide an optimal pH in the matrix to allow an efficient enzyme conversion of these metabolites. Although hydrogenosomes bear only a single membrane, in functional terms they may represent the anaerobic equivalent of mitochondria, which are absent in anaerobic eukaryotes such as *Neocallimastix* sp. L2.

The hydrogenosomal ATPase probably functions as proton-translocating ATPase, with its hydrolytic site located inside the hydrogenosome. Proton pumping from the matrix of the hydrogenosome into the cytosol results in the generation of a pH gradient across the hydrogenosomal membrane, inside alkaline *v.* outside. Just like hydrogenosomes from *Neocallimastix* sp. L2, hydrogenosomes from *Trichomonas vaginalis* have also been reported to have an alkaline internal pH (Yarlett *et al.*, 1987). The observation that isolated hydrogenosomes were able to create a pH gradient without the addition of external ATP can be explained by the presence of metabolic activity and residual ATP or ADP in the organelles. In this respect, the hydrogenosomal adenylate kinase enables the production of ATP and AMP from ADP (Marvin-Sikkema *et al.* 1993*b*), thereby maintaining an ATP pool inside the organelles. However, due to the lability of the isolated hydrogenosomes we have not undertaken attempts to deplete the hydrogenosomes extensively from residual nucleotides by the use of glucose/hexokinase, and apyrase (to degrade ADP into adenosine and inorganic phosphate). The lack of a detectable $\Delta\psi$ suggests that the ion-permeability of the hydrogenosomal membrane is relatively high. On the other hand, we observed that the K^+-ionophore valinomycin is a potent inhibitor of H_2 production from malate. However, hydrogenosomes of *Neocallimastix* sp. L2 metabolize malate only under anaerobic conditions, while Δp measurements have been performed in resting hydrogenosomes under aerobic conditions.

With respect to its characteristics and sensitivity to various ATPase inhibitors, the hydrogenosomal ATPase is difficult to classify. The pH optimum and Mg^{2+} or Mn^{2+} dependency resembles the properties of mitochondrial ATPases (Pedersen & Carafoli, 1987) of aerobic eukaryotes, the microbody ATPase of the yeast *Hansenula polymorpha* (Douma *et al.*, 1987) and ATPases in anaerobic eukaryotes *Tritrichomonas*

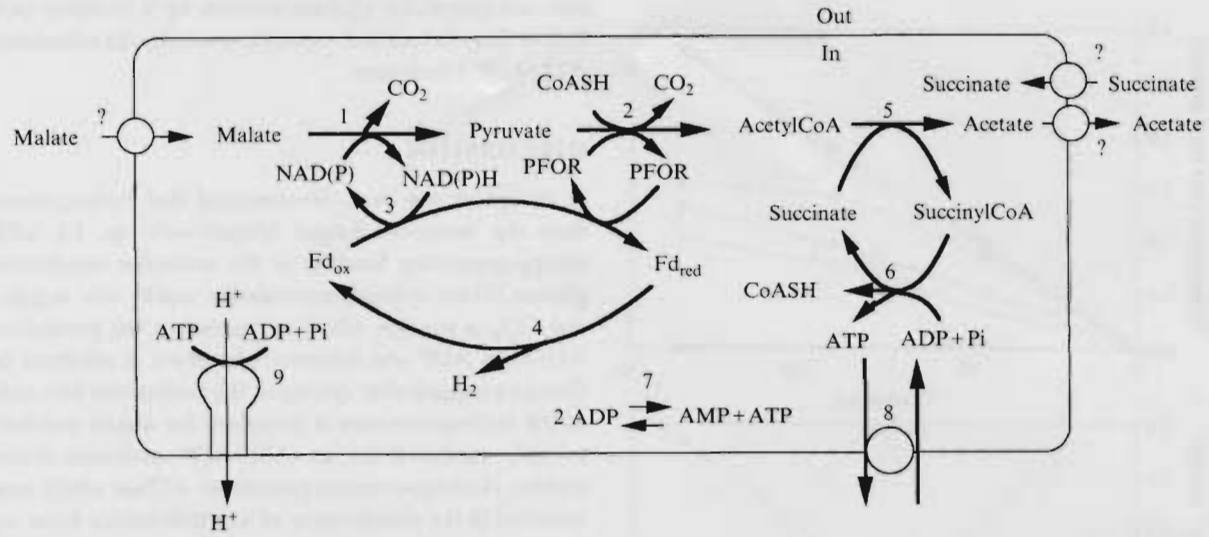

Fig. 5. Scheme of the hydrogenosome of *Neocallimastix* sp. L2. 1, 'malic enzyme'; 2, pyruvate:ferredoxin oxidoreductase; 3, NAD(P)H:ferredoxin oxidoreductase; 4, hydrogenase; 5, acetate:succinate CoA transferase; 6, succinate thiokinase; 7, adenylate kinase; 8, ATPase; 9, ADP/ATP translocase.

foetus (Lloyd, Lindmark & Müller, 1979) and *Trichomonas vaginalis* (Turner & Lushbaugh, 1991). Although the latter two organisms contain hydrogenosomes, ATPase activity in the isolated organelles appeared to be absent (Lloyd, Lindmark & Müller, 1979; Turner & Luschbaugh, 1991). Unlike the mitochondrial ATPases, the hydrogenosomal ATPase is not affected by DCCD, oligomycin or azide, while it is sensitive to DES, a typical inhibitor of E_1E_0 ATPases (P-type). P-type ATPase normally occurs in cell plasma membranes of fungi (Goffeau & Slayman, 1981). On the other hand, unlike P-type ATPases (Bowman, Siebers & Altendorf, 1988) the hydrogenosomal ATPase is not affected by vanadate.

Two lines of evidence based on the effect of the typical inhibitors of the mitochondrial ATP/ADP translocase, i.e. bongkrekic acid and carboxyatractylate, suggest that a similar system operates in hydrogenosomes. These compounds lower the accessible ATPase activity measured with intact hydrogenosomes, and block H_2 production from malate, which depends on the external supply of nucleotides. The ATP/ADP translocase may function in the export of ATP, which is produced inside the hydrogenosome by substrate-level phosphorylation. The presence of such a system in hydrogenosomes lends further support to the notion that these organelles are functionally related to mitochondria, which are typically found in aerobic eukaryotes.

Although ATPase activity was absent in *T. vaginalis* (Turner & Luschbaugh, 1991) and *Trit. foetus* (Lloyd *et al.*, 1979), both were able to produce ATP from ADP during the anaerobic fermentation of pyruvate (Steinbüchel & Müller, 1986). Under aerobic conditions *Trit. foetus* hydrogenosomes were shown to act as respiratory organelles, with pyruvate as substrate, but only in the presence of ADP, inorganic phosphate and succinate. This process was inhibited by atractylate, but as uncouplers of mitochondrial of mitochondrial oxidative phosphorylation were ineffective, the observed effects were not identical to mitochondrial respiration (Čerkasov *et al.*, 1978).

Our current working model on the function of hydrogenosomes in the metabolism of malate and the role of compartmentalization is shown in Fig. 5. Isolated hydrogenosomes from *Neocallimastix* sp. L2 produce acetate and H_2 from malate (Marvin-Sikkema *et al.*, 1993*b*; this paper). The inability of the hydrogenosomes to produce H_2 and acetate from pyruvate presumably relates to an inability to transport pyruvate, since hydrogenosomes of *Trit. foetus* and *T. vaginalis* are well able to metabolize pyruvate (Steinbüchel & Müller, 1986). Pyruvate is formed in the reaction catalysed by 'malic enzyme' mediating the decarboxylation of malate. The next step is catalysed by pyruvate:ferredoxin oxidoreductase and acetate:succinate CoA transferase mediating the conversion of pyruvate into acetate coupled to the synthesis of ATP from ADP and inorganic phosphate. Since this enzymic step is reversible, efficient energy generation requires a high pyruvate and ADP concentration in the hydrogenosomal lumen, and a rapid expulsion of ATP and acetate. The ATP/ADP translocase may serve to regulate the matrix nucleotide pools to permit rapid metabolism of malate. The effects of P^1P^5-di(adenosine 5'-) pentaphosphate, bongkrekic acid and carboxyatractylate may directly relate to the nucleotide dependency of this process. The lowered H_2 production in the presence of CO_2 is possibly caused by a dependence of 'malic enzyme' and/or pyruvate:ferredoxin oxidoreductase on the P_{CO_2} (Marvin-Sikkema *et al.*, 1993*b*). Succinate may promote H_2 production from malate as it can serve as an acceptor for the CoA moiety of acetylCoA in the CoA transferase reaction (Marvin-Sikkema *et al.*, 1993*b*). This implies that succinate is transported across the hydrogenosomal membrane, as succinate is not produced inside the hydrogenosomes (Marvin-Sikkema *et al.*, 1993*b*).

In addition to the roles described above, ATP may also be required to maintain a Δp ($\Delta\psi$ and ΔpH) across the hydrogenosomal membrane. Δp is an important parameter in the reactions mediated by the hydrogenosomes, as iono- and protonophores are strong inhibitors of H_2 production from malate. The mechanism by which these compounds act may be either direct or indirect. Since net charge transfer is

involved in the stoichiometric exchange of cytosolic ADP and matrix ATP, steady-state concentrations may exist of the magnitude of the $\Delta\psi$. On the other hand, since ΔpH affects the absolute pH in the matrix, enzymic activity may indirectly be modulated by Δp. Furthermore, Δp may function as a driving force for the uptake and/or expulsion of other metabolites such as malate, acetate and succinate (see below). From the effect of valinomycin, we anticipate that the $\Delta\psi$ is an important parameter in the production of H_2 from malate. It should, however, be emphasized that, in spite of the fact that we were unable to demonstrate a $\Delta\psi$ in resting hydrogenosomes, such measurements have not yet been made under anaerobic and malate-metabolizing conditions.

Cell compartmentalization allows the separation of metabolic reactions that would otherwise lead to futile cycles. In some bacteria, decarboxylation is directly coupled to an energy-yielding process. Membrane-bound decarboxylases have been found that couple the enzymic reaction to the translocation of Na^+ across the cytoplasmic membrane (Dimroth, 1987). The 'malic enzyme' of hydrogenosomes of *Neocallimastix* sp. L2 is soluble, and not associated with the membrane (Marvin-Sikkema, unpublished data). Coupled transport of malate into and excretion of acetate out of the hydrogenosomes may further add to the generation of metabolic energy. This process may resemble that found in other decarboxylation reactions in bacteria such as oxalate/formate antiport in *Oxalobacter formigenes* (Anantharam, Allison & Maloney, 1989), malate/lactate exchange in *Lactococcus lactis* (Poolman *et al.*, 1991) and histidine/histamine exchange in *Lactobacillus buchneri* (Molenaar *et al.*, 1993). Stoichiometric exchange of the malate anion, Mal^{2-}, for acetate will result in the generation of a $\Delta\psi$, inside negative. This reaction would be tightly coupled to H_2 production as it is driven by the concentration gradients of malate (high outside) and acetate (high inside). Further work will be needed to elucidate the mechanisms of transport across the hydrogenosomal membrane. Such studies require the use of membrane vesicles to uncouple the transport steps from metabolic activities in the hydrogenosomal lumen, and will allow us to define whether transport and compartmentalization contribute to the metabolic energy generation during anaerobic growth of *Neocallimastix* sp. L2.

In addition to its role in the translocation of substrates and/or products across the membrane (LaNoue & Schoolwerth, 1984; Sato, Uhsumi & Ankaru, 1984), Δp may also have a major role in the import of proteins by organelles in eukaryotic cells (Verner & Schatz, 1988). Δp may have similar functions in hydrogenosomes, but nothing is known about the biogenesis of these organelles in anaerobic fungi. Many questions remain such as: do hydrogenosomes contain DNA, and can they replicate autonomously and import proteins just as mitochondria? Attempt to demonstrate the presence of Hsp60 (GroEL) and Hsp70 (DnaK) homologues in hydrogenosomes from *Neocallimastix* sp. L2 by immunological means have so far failed (F.-U. Hartl, personal communications). Anaerobic fungi possibly evoked from aerobic organisms, and adapted to an anaerobic way of life in the digestive tract of herbivorous mammals by dramatic changes in their metabolism. Preservation of all necessary mitochondrial functions into a single-walled organelle may have been one of the adaptations, and studies of the hydrogenosomal biogenesis may further help in understanding the evolutionary origin of hydrogenosomes from anaerobic fungi.

REFERENCES

Akin, D. E., Lyon, C. E., Windham, W. R. & Rigsby, L. L. (1989). Physical degradation of lignified stem tissues by ruminal fungi. *Applied and Environmental Microbiology* **55**, 611–616.

Anantharam, V., Allison, M. J. & Maloney, P. C. (1989). Oxalate:formate exchange. The basis for energy coupling in *Oxalobacter*. *Journal of Biological Chemistry* **264**, 7244–7250.

Bowman, E. J., Siebers, A. & Altendorf, K. (1988). Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 7972–7976.

Čerkasov, J., Čerkasovová, A., Kulda, J. & Vilhelmová, D. (1978). Respiration of hydrogenosomes of *Tritrichomonas foetus*. 1. ADP-dependent oxidation of malate and pyruvate. *Journal of Biological Chemistry* **253**, 1207–1214.

Declerk, P. J. & Müller, M. (1986). Hydrogenosomal ATP:AMP phosphotransferase of *Trichomonas vaginalis*. *Comparative Biochemistry and Physiology* **88B**, 575–580.

Dimroth, P. (1987). Sodium ion transport decarboxylases and other aspects of sodium ion cycling in bacteria. *Microbiological Reviews* **51**, 320–340.

Douma, A. C., Veenhuis, M., Sulter, G. J. & Harder, W. (1987). A proton-translocating adenosine triphosphatase is associated with the peroxisomal membrane of yeasts. *Archives of Microbiology* **147**, 42–47.

Gerritse, J., Schut, F. & Gottschal, J. C. (1990). Mixed chemostat cultures of obligately aerobic and fermentative or methanogenic bacteria grown under oxygen-limiting conditions. *FEMS Microbiology Letters* **66**, 87–94.

Goffeau, A. & Slayman, C. W. (1981). The proton-translocating ATPase of the fungal plasma membrane. *Biochimica et Biophysica Acta* **639**, 197–223.

Gutman, I. & Wahlefeld, A. W. (1974). L-malate determination with malate dehydrogenase and NAD. In *Methods in Enzymatic Analysis* (ed. H. U. Bergmeyer), pp. 1585–1589. Verlag Chemie: Weinheim.

Hellingwerf, K. J. & Konings, W. N. (1980). Kinetic and steady-state investigations of solute accumulation in bacterial membranes by continuous monitoring of the effluent by flow-dialysis experiments. *European Journal of Biochemistry* **106**, 431–437.

LaNoue, K. F. & Schoolwerth, A. C. (1984). Metabolite transport in mammalian mitochondria. In *Bioenergetics* (ed. L. Enrster), pp. 221–268. Elsevier Scientific: Amsterdam.

Lloyd, D., Lindmark, D. G. & Müller, M. (1979). Adenosine triphosphatase activity of *Tritrichomonas foetus*. *Journal of General Microbiology* **115**, 301–307.

Marvin-Sikkema, F. D., Richardson, A. J., Stewart, C. S., Gottschal, J. C. & Prins, R. A. (1990). Influence of hydrogen consuming bacteria on cellulose degradation by anaerobic fungi. *Applied and Environmental Microbiology* **56**, 3793–3797.

Marvin-Sikkema, F. D., Lahpor, G. A., Kraak, M. N., Gottschal, J. C. & Prins, R. A. (1992). Characterization of an anaerobic fungus from llama faeces. *Journal of General Microbiology* **138**, 2235–2241.

Marvin-Sikkema, F. D., Kraak, M. N., Veenhuis, M., Gottschal, J. C. & Prins, R. A. (1993*a*). The hydrogenosomal enzyme hydrogenase from the anaerobic fungus *Neocallimastix* sp. L2 is recognized by antibodies, directed against the C-terminal microbody protein targeting signal SKL. *European Journal of Cell Biology* **61**, 86–91.

Marvin-Sikkema, F. D., Pedro Gomes, T. M., Grivet, J.-P., Gottschal, J. C. & Prins, R. A. (1993*b*). Characterization of hydrogenosomes and their role in glucose metabolism of *Neocallimastix* sp. L2. *Archives of Microbiology* (in press).

Milne, A., Theodorou, M. K., Jordan, M. G. C., King-Spooner, C. & Trinci, A. P. J. (1989). Survival of anaerobic fungi in feces, in saliva, and in pure culture. *Experimental Mycology* **13**, 27–37.

Molenaar, D., Bosscher, J. S., ten Brink, B., Driessen, A. J. M. & Konings, W. N. (1993). Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*. *Journal of Bacteriology* **175**, 2864–2870.

Müller, M. (1988). Energy metabolism of protozoa without mitochondria. *Annual Review of Microbiology* **42**, 465–488.

Nanninga, H. J., Drent, W. J. & Gottschal, J. C. (1986). Major differences between glutamate-fermenting species isolated from chemostat enrichments at different dilution rates. *FEMS, Microbiology and Ecology* **38**, 321–29.

Orpin, C. G. & Joblin, K. N. (1988). The rumen anaerobic fungi. In *The Rumen Microbial Ecosystem* (ed. P. N. Hobson), pp. 129–151. Elsevier Applied Science: London.

Pedersen, P. L. & Carafoli, E. (1987). Ion motive ATPases. I. Ubiquity, properties, and significance to cell function. *Trends in Biochemical Sciences* **12**, 146–150.

Poolman, B., Molenaar, D., Smid, E. J., Ubbink, T., Abee, T., Renault, P. P. & Konings, W. N. (1991). Malolactic fermentation: electrogenic malate uptake and malate/lactate antiport generate metabolic energy. *Journal of Bacteriology* **173**, 6030–6037.

Sato, T., Ohsumi, Y. & Ankaru, Y. (1984). Substrate specificities of active transport systems for amino acids in vacuolar-membrane vesicles of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **259**, 11505–11508.

Steinbüchel, A. & Müller, M. (1986). Anaerobic pyruvate metabolism of *Tritrichomonas foetus* and *Trichomonas vaginalis* hydrogenosomes. *Molecular and Biochemical Parasitology* **20**, 57–65.

(*Accepted 13 July 1993*)

Turner, A. C. & Luschbaugh, W. B. (1991). Three aspecific ATPases in *Trichomonas vaginalis*. *Comparative Biochemistry and Physiology* **100B**, 691–696.

Verner, K. & Schatz, G. (1988). Protein translocation across membranes. *Science* **241**, 1307–1313.

De Vrij, W., Driessen, A. J. M., Hellingwerf, K. J. & Konings, W. N. (1986). Measurements of the proton motive force generated by cytochrome c oxidase from *Bacillus subtilis* in proteoliposomes and membrane vesicles. *European Journal of Biochemistry* **156**, 431–440.

Widnell, C. C. (1974). Purification of rat liver 5′-nucleotidase as a complex with sphingomyelin. *Methods in Enzymology* **32**, 368–374.

Williams, A. G. & Orpin, C. G. (1987). Polysaccharide-degrading enzymes formed by three anaerobic fungi grown on a range of carbohydrate substrates. *Canadian Journal of Microbiology* **33**, 418–426.

Yarlett, N., Rowlands, C., Evans, J., Yarlett, N. C. & Lloyd, D. (1987). Nitroimidazole derived radicals detected by electron spin resonance in hydrogenosomal and cytosolic fractions from *Trichomonas vaginalis*. *Molecular and Biochemical Parasitology* **24**, 255–261.

Yarlett, N., Orpin, C. G., Munn, E. A., Yarlett, N. C. & Greenwood, C. A. (1986). Hydrogenosomes in the rumen fungus *Neocallimastix patriciarum*. *Biochemical Journal* **236**, 729–739.